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THE EFFECTS OF HYDRAZINES ON NEURONAL EXCITABILITY

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Hydrazines are toxic compounds with military and industrial applications including use as missile propellants for aircraft (i.e. the F-16 and space vehicles). Recurrent exposure from routine storage, use, and disposal hydrazines makes their toxic effects on the nervous system important. Exposure to hydrazine can result in status epilepticus and eventual respiratory collapse. Acute exposure can produce repeated tonic-clonic seizures in both animal and man. This project has continued to direct its effort in understanding the molecular mechanism by which hydrazines may produce their neuronal excitatory effects. Our investigation focuses on the effects of hydrazine on electrophysiological properties of identified neurons in the invertebrate Hermissenda Crassicornis. We have documented that hydrazines increase neuronal excitability in the LP-1 neuron of this nudibranch mollusc. Hydrazines also increase the rate of sustained repetitive firing in this system. We will study molecular mechanism mediating the effects of hydrazine on increased neuronal firing in isolated neurons. Specific anticonvulsant drugs may have potential benefit in blocking the excitable effects of hydrazine on neuronal activity.

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Grant Number: AFOSR-87-0235

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ABSTRACT

Hydrazines are toxic compounds which have numerous military and industrial applications including their use in missile propellants and advanced aircraft such as the F-16 and space vehicles. Because of the recurrent exposure due to routine storage use and disposal of these compounds, understanding their toxic effects on the nervous system is important in the aerospace field. Toxic exposure to hydrazine can result in status epilepticus and eventual respiratory collapse. Acute hydrazine exposure can produce repeated tonic-clonic seizures in animals and man. This project has continued to direct its effort in understanding the molecular mechanism by which hydrazines may produce their neuronal excitatory effects. We have continued and expanded our investigation of the effects of hydrazine on specific electrophysiological properties on identified neurons in the invertebrate Hermissenda Crassicornis. Our studies have documented that hydrazines increase neuronal excitability in the LP-1 neuron of this nudibranch mollusc. Studies have been directed at establishing the technical capability of investigating the effects of hydrazines on the rate of sustained repetitive firing. It has been shown that hydrazines increase the rate of sustained repetitive firing in this system. Studies have also been initiated to elucidate the molecular mechanism mediating the effects of hydrazine on increased neuronal firing in isolated neurons. It has also been determined that specific anticonvulsant drugs may have potential benefit in blocking the excitable effects of hydrazine on neuronal activity. The project has attained the initial specific aims and preliminary goals developed for this phase of the project period.

RESEARCH OBJECTIVES

The overall major objective of this study is to determine the mechanisms by which hydrazines increase neuronal excitation and cause seizure activity. We have initiated studies to examine the effects of hydrazines on specific ion conductances in neurons in the nudibranch mollusc, Hermissenda Crassicornis. These studies are also directed at obtaining an insight into the effects of these toxic compounds on neuronal excitability and seizure discharge. The central hypothesis that is being tested in this project is that hydrazines increase neuronal firing by altering specific membrane currents.

During this year of the project, we have made substantial progress in obtaining our short-term goals. The project has characterized the effects of hydrazine on increased neuronal firing and examined specific ion currents in the identified LP-1 neuron. Thus, we have been successful in characterizing the effects of hydrazine on sustained repetitive firing (SRF) and spike frequency adaptation (SFA) in Hermissenda LP-1 neurons. This accomplishes the major short-term goal of our project.

Studies have also been initiated during this first year to characterize specific membrane currents in the LP-1 Hermissenda neuron. The currents that are being investigated include I_A, I_C, I_{Ca}, I_{Na}, I_K. These studies are being conducted with two electrode voltage clamp techniques. Following the characterization and identification of these currents, studies will next be initiated to determine the effects of hydrazines on specific ion currents under the voltage clamp technique.

Initial experiments have also been directed at accomplishing the longer-term goals of the project by developing significant preliminary data and feasibility study information. We have investigated the effects of specific anticonvulsants on the excitability of the LP-1 neuron in Hermissenda. We have determined the conditions by which the benzodiazepines and other neuroleptic compounds can be utilized to decrease repetitive firing and alter other parameters

of these cells. These experiments include the development of specific techniques for the intracellular micro-injection of these compounds or for direct bath application. The solubility of these compounds and the use of appropriate controlled bathing medium have been studied and we now are capable of testing these agents on LP-1 physiology in the presence and absence of hydrazine. These studies will allow us to conduct the determination of the effects of these compounds on hydrazine-produced effects in this neuron. Initial studies to investigate the effects of hydrazines on calcium regulated ion conductances in identified neurons have also been initiated. Potassium currents are major regulators of neuronal excitability and, if they are involved in the toxic effects, we have developed the technology to determine this in the second and third years of this study.

RESEARCH STATUS REPORT

During the first year of this project we have made significant progress in obtaining the specific aims outlined in the original proposal. The program research is on target and we have completed the initial research and characterization of our system to allow us to continue with the more involved specific aims of the longer term goals in the next two years of the project.

Effects of Hydrazine on Spike Frequency Adaptation in Hermissenda Neurons

Spike frequency adaptation (SFA) is a cellular mechanism that is utilized by cells to reduce the frequency of firing when a constant current stimulus is applied to the cell. This process has been well investigated as a mechanism for regulating seizure activity. It has been shown that anticonvulsant drugs that enhance SFA are potent anticonvulsants against generalized tonic-clonic seizures, the type of seizures produced by toxic hydrazine exposure. Thus, modulation of SFA may be an important mechanism by which anticonvulsants produce

their effects against generalized tonic-clonic seizures. In addition, since hydrazines induce tonic-clonic seizures, it is reasonable to investigate their effects on SFA in identified neurons. Alterations in SFA firing could have the opposite effect of the anticonvulsant drugs and account for the convulsant effects of hydrazine in man and animals.

During this project period we have expanded our studies on the effects of hydrazines on spike frequency adaptation. These studies were performed on the LP-1 neuron in Hermissenda. This research utilized both intracellular recording and voltage clamp techniques. Numerous experiments were performed by impaling the LP-1 neuron with a single microelectrode. In these experiments constant current is applied to the cell and spike trains are produced in response to stimulation. These spike trains are recorded using a chart recorder or computer data system.

We have determined that in the presence of as low as 10 micromolar hydrazine, there is a significant effect on spike frequency adaptation. Hydrazine produced a reproducible reduction of spike frequency adaptation. These studies document that hydrazine limits spike frequency adaptation in the LP-1 neuron. These results are shown in Figure 1. The effects of hydrazine clearly document that this compound can block the cells ability to turn off repetitive spiking. Thus, a neuron exposed to hydrazine becomes excitable. Hydrazine produced the same effects on this neuron as several known convulsants. The reversibility of the hydrazine effects on SFA indicates that the concentrations used are not simply killing or injuring the cell resulting in increased neuronal excitability. These experiments have been performed under conditions where the hydrazine effect is not lethal to the cell and the cell can make a complete electrophysiological recovery as shown in Part C of the Figure 1.

These experimental results have been conducted with numerous appropriate controlled experiments. This research now establishes that hydrazines can behave as other convulsant drugs and produce increased neuronal firing as shown in Figure 1. We have also demonstrated

in our laboratory that anticonvulsant drugs such as Diazepam, Medazepam, and Phenytoin can block sustained repetitive firing and enhance SFA. These compounds dramatically enhance SFA. Experiments are currently underway to determine if these compounds can block the specific excitatory effects of hydrazine on the LP-1 neuron.

These experiments are technically difficult to perform because they require the mixing of both hydrazines and other drugs in the bathing solution. This must be done under conditions to maintain the solubility of the anticonvulsant drugs while simultaneously applying hydrazine. Solubilizing both of these compounds in appropriate concentrations in artificial sea water has been technically difficult, but we have now produced preliminary data that suggests that we have been successful in developing this technique. Thus, we are now investigating the effects of anticonvulsant drugs in blocking the effects of hydrazine on repetitive firing and spike frequency adaptation. Figures 2 and 3 document the effects of specific anticonvulsant drugs on sustained repetitive firing in identified neurons in Hermissenda. These experiments document that we now are ready to initiate the next phase of this research and determine which compounds can regulate the effects of hydrazine on neuronal excitability.

In summary, we have accomplished the major specific aims of this first project period. We have established that hydrazine clearly reduces SFA. The effect of hydrazine was clearly reversible and was shown to effect SFA in the same manner as other convulsant drugs. We have also conducted the initial experiments in this invertebrate model to test the effect of anticonvulsant drugs on the effects of hydrazine on this system. We have documented that anticonvulsant drugs can regulate SFA and are now initiating experiments to determine their action on hydrazine's excitable effect on excitability.

Characterization of Membrane Currents in Identified Hermissenda Neurons

During the first year of this project we have also been successful in characterizing

specific membrane currents in identified Hermissenda neurons. We have also investigated spike frequency adaptation and sustained repetitive firing in other identified neurons besides LP-1. These studies are important because specific neurons have variable excitatory properties. Thus, it is important to determine whether the effects of hydrazine seen on the LP-1 neuron are representative of other neurons in this preparation. We have obtained data that clearly documents our preliminary findings that we can measure and characterize the currents I_A, I_{Ca}. We have measured these currents successfully in the LP-1 neurons in our laboratory. We have also investigated other specific ion conductances including I_K, I_{Na}, I_C. These currents are clearly present in the LP-1 neuron and we have obtained initial evidence that establishes that we can isolate these currents under voltage clamp procedures.

The I_A current is called the "A" current. The A current is an outward voltage-dependent potassium current which is blocked by three millimolar 4-aminopyridine. Thus, we have been successful in evaluating this current in a sodium-free medium that contains cobalt or cadmium to block the inward flow of sodium or calcium. This also inhibits the I_C current. Thus, under conditions where we can block most other ion flows, we can isolate the I_A current. In addition, the A current has been shown in the LP-1 neuron to be inactivated at resting potential. Thus, this current can be measured in a two-pulse paradigm in which the membrane potential is a pre-step to a hyperpolarizing voltage.

The A current is interesting because it exhibits both calcium dependence as well as voltage dependent activation. It is our hypothesis that the A current may be an excellent candidate in mediating some of the effects of hydrazine on neuronal excitability. Now that we have established the initial parameters of these currents in the LP-1 and other neurons in Hermissenda ganglia, we are ready to initiate the second phase of our studies looking at the effects of hydrazines on this specific current.

We have also made considerable progress in characterizing the inward calcium current,

We have also made considerable progress in characterizing the inward calcium current, I_{Ca}. The calcium current is measured as peak inward current in a sodium-free sea water solution containing three millimolar, 4-aminopyridine and TEAE (100 millimolar) which blocks both the I_A and I_K currents. Barium is utilized to replace calcium as the current carrier to eliminate the effects of calcium on the potassium currents. We have been successful in clamping LP-1 neurons and holding voltage of approximately -50 millivolts. Test pulses were given to elicit the calcium current for durations of 500 milliseconds. We have determined that the I_{Ca} exhibits calcium dependent inactivation in the LP-1 neuron. The studies initiated in this first year of research have demonstrated that we now have established parameters to study this current and the effect of hydrazine on this isolated calcium current.

We have been successful in establishing and characterizing extensively two specific ion currents and have begun studies on sodium and other currents. It is essential to rigorously establish the parameters of these currents in the identified neuron so that careful analysis of the effects of hydrazine can be determined. It is important to exclude artifactual effects secondary to changes in cell environment. Thus, we are being extremely careful to rigorously define our electrophysiological system before characterizing the effects of hydrazine. We have accomplished the initial goals of our project and are now ready to initiate the hydrazine studies on these identified currents. We plan to expand these studies to investigate other currents especially if hydrazine does not effect I_A or I_{Ca} currents.

Phasing of Research Projects

During this first year of this research effort, we have made exceptional progress in our ability to bring together necessary personnel and equipment to initiate these studies. We have successfully documented the effects of hydrazine on sustained frequency adaptation and began experiments to characterize this effect on specific ion currents. We have also successfully

developed the procedures for studying the interaction between hydrazine and other known anticonvulsant and convulsant drugs. Thus, we have accomplished the major objectives of the first year of the project. In addition, we have also established the technical base to extend this research into the investigation of other specific ion currents and carefully characterize the effects of hydrazine on these currents in the LP-1 neuron and other cells in this mollusc. These studies will be important in providing the first electrophysiological data on the effects of hydrazine on ion currents.

Although these studies are performed on an invertebrate system, it is also essential that this research be extended to vertebrate neurons. We have successfully developed the technology to prepare and maintain vertebrate culture neurons isolated from fetal hippocampus and spinal cord. We are initiating studies to perform and evaluate sustained frequency adaptation, sustained repetitive firing, and specific ion currents in these vertebrate cells. We have included this research in our long term goals and hope that within the second or third year of this project to be able to extend our studies in the mollusc system to the vertebrate neurons in culture. This is a significant advance in our research effort and has been brought about by the rapid progress that we have made on this project. These studies will significantly expand our objectives and may provide important comparisons between invertebrate and vertebrate neurons. The demonstration of the effect of hydrazine on these cells with rigorous electrophysiological studies may provide the initial framework for understanding the excitable effect of this compound on neuronal function.

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Professional Personnel Engaged in this Study

Dr. Robert J. DeLorenzo has acted as the Principal Investigator in studying the effects of hydrazines on neuronal excitability. In addition, Dr. Robin Forman has participated as a co-investigator. Dr. Forman has been responsible for specific electrophysiological experiments and for assisting in the development of the studies on the anticonvulsant effects on isolated LP-1 neurons. Dr. William Taft has also been involved in assisting with the anticonvulsant and convulsant drug studies on isolated neurons. We have also initiated a collaborative interactions of Dr. Sompong Sombati and Dr. William Anderson who have developed the neuronal cells in culture. Dr. Sombati, in collaboration with Dr. Forman and Dr. DeLorenzo, has initiated studies on these vertebrate neurons in culture and has begun characterization of specific ion currents in these cells. This collaboration has developed rapidly and it is presumed that in the second and third years of this project, Dr. Sombati, in collaboration with Dr. Forman and Dr. DeLorenzo, will expand our studies to include the effects of hydrazine on these cultured vertebrate neurons. Ben Churn, Graduate student in the Department of Pharmacology, has also assisted in some of the basic biochemical studies related to this project. In addition, Bruce Puryear, a medical student at the Medical College of Virginia, has

also been involved in research on this project.

Inventions and Patents

No specific inventions or patents have been developed from this research period.

Summary

During the first year of this research project, we have made considerable progress in directing our efforts at studying the effects of hydrazine on neuronal excitability. The initial studies have been exciting and have led to research that will provide the first documentation of the effects of this compound on isolated neurons. In addition, the ability to incorporate experiments into this project that will also allow us to investigate the effects of hydrazine on vertebrate neurons considerably expands the scope of our initial goals. Development of this technology in our laboratory over the last year will greatly enhance the application and feasibility of these studies. We plan to initiate studies in vertebrate neurons during the second and third years of the project. We have also been successful in characterizing specific ion currents and their sensitivity to specific anticonvulsant and convulsant drugs. These studies will allow us now to more rigorously investigate mechanisms that may block the toxic effects of hydrazines on the nervous system.

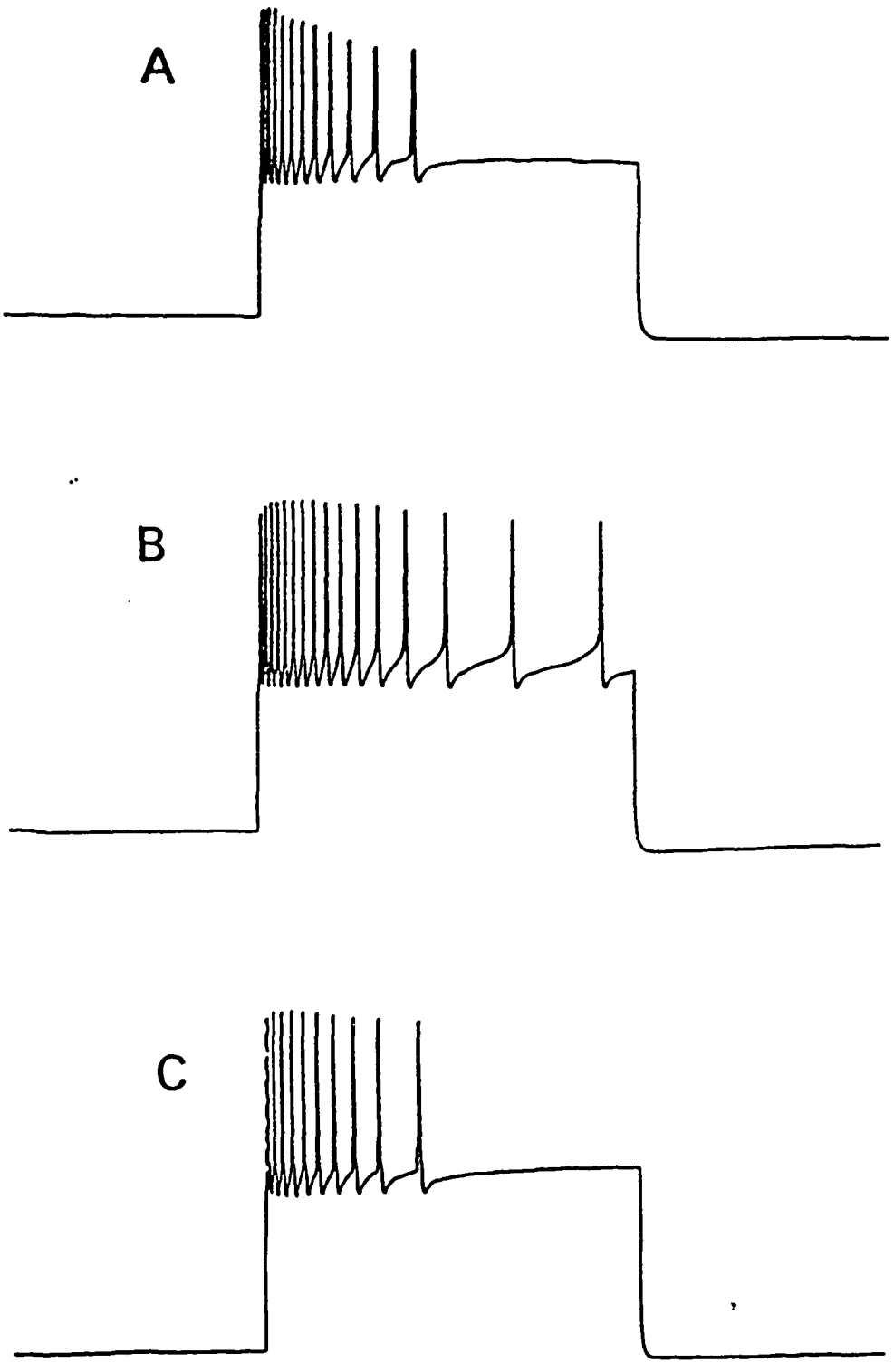

Figure 1. Effect of hydrazine on spike frequency adaptation (SFA) in a Hermissenda LP1 neuron. SFA is the process by which neurons limit their firing rate in response to a constant stimulus, thereby limiting neuronal excitability. Traces were recorded from the same cell before (A) and 3 min after (B) 10 mM hydrazine application. The HZ effect was reversible on washout (C). Adaptation of repetitive firing shown in (A) was markedly reduced by HZ resulting in increased firing (B).

Figure 2.

Effect of intracellular pressure ejection of a water-soluble BZ, medazepam (MDZ), on repetitive firing in an identified Hermissenda neuron. A) Before MDZ ejection. B) 2 min post-ejection. C) 5 min post-ejection. Action potential firing elicited by a 1.5 nA, 10 sec depolarizing current pulse. Pressure ejection of MDZ produced a significant decrease in SRF. Calibration bar: 20 mV.

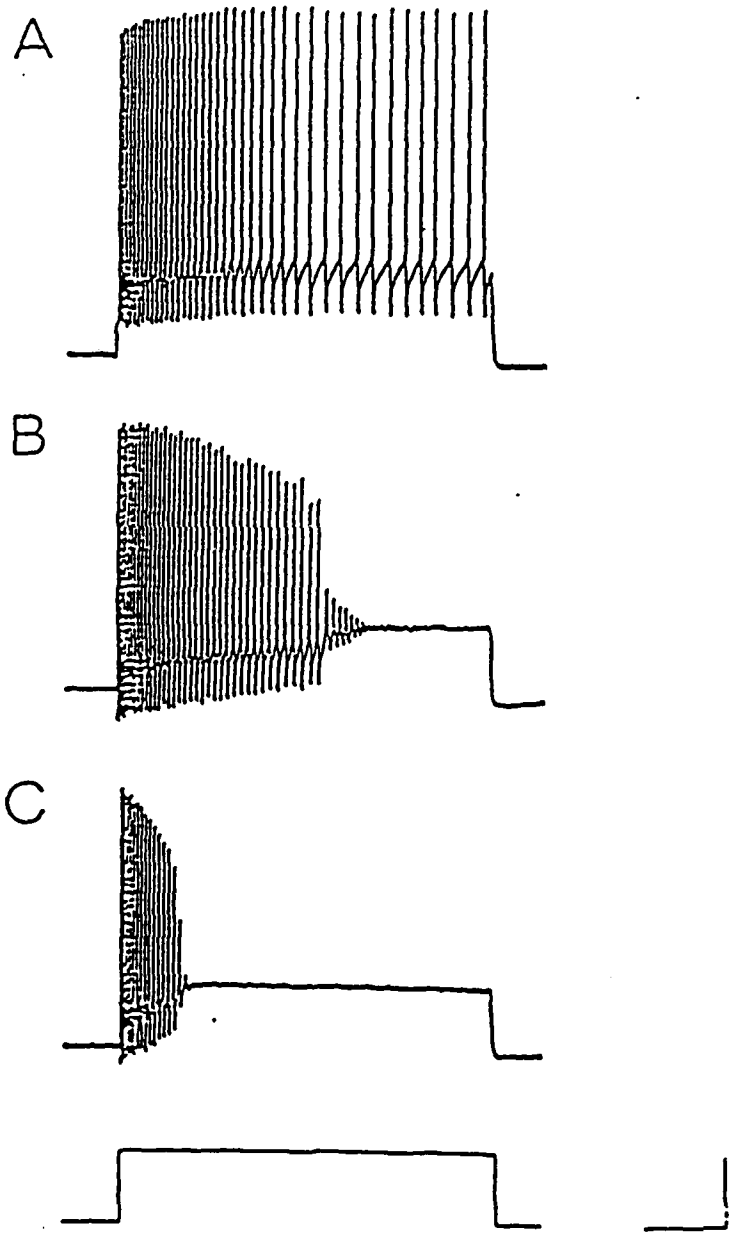

Figure 3.

Effect of bath application of flunitrazepam (FNZ) on spike frequency adaptation (SFA) in an identified Hermissenda neuron. A) Before application of FNZ. B) 2 min following FNZ. C) 5 min following FNZ. Activity was elicited by a 1.5 nA, 10 sec depolarizing current pulse. Bath application of FNZ produced a dramatic enhancement of SFA. Calibration bar: 20 mV.